

Pharmaceutical composition of hydrophobically modified hedgehog proteins and  
their use

The invention concerns a pharmaceutical composition of hydrophobically modified hedgehog proteins and their use, in particular for the local release of these proteins on bones or cartilage.

5 Hedgehog (hh) proteins are understood as a family of secreted signal proteins which are responsible for the formation of numerous structures in embryogenesis (J.C. Smith, Cell 76 (1994) 193 - 196, N. Perrimon, Cell 80 (1995) 517 - 520, C. Chiang et al., Nature 83 (1996) 407, M.J. Bitgood et al., Curr. Biol. 6 (1996) 296, A. Vortkamp et al., Science 273 (1996) 613, C.J. Lai et al., Development 121 (1995) 2349). During its biosynthesis a 20 kD N-terminal domain and a 25 kD C-terminal domain are obtained after cleavage of the signal sequence and autocatalytic cleavage. In its natural form the N-terminal domain is modified with cholesterol (J.A. Porter et al., Science 274 (1996) 255 - 259). In higher life-forms the hh family is composed of at least three members namely sonic, indian and desert hh (shh, Ihh, Dhh; M. Fietz et al., Development (Suppl.) (1994) 43 - 51). Differences in the activity of hedgehog proteins that were produced recombinantly were observed after production in prokaryotes and eukaryotes (M. Hynes et al., Neuron 15 (1995) 35 - 44 and T. Nakamura et al., Biochem. Biophys. Res. Comm. 237 (1997) 465 - 469).

20 Hynes et al. compare the activity of hh in the supernatant of transformed human embryonic kidney 293 cells (eukaryotic hh) with hh produced from E. coli and find a four-fold higher activity of hh from the supernatants of the kidney cell line. The reason for this increased activity has been discussed to be a potential additional accessory factor which is only expressed in eukaryotic cells, a post-translational modification, a different N-terminus since the hh isolated from E. coli contains 50 % of a hh form which carries two additional N-terminal amino acids (Gly-Ser) or is shortened by 5 - 6 amino acids, or a higher state of aggregation (e.g. by binding to nickel agarose beads).

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It is known from Yang et al., Development 124 (1997) 4393-4404 that high local hedgehog concentrations must prevail over a period of at least 16 h at the site of action in the body for a pharmaceutically effective in vivo activity. The carrier system for this described by Yang et al. i.e. the hedgehog-loaded chromatography medium affigel CM, the Ni agarose described by Marti et al., in Nature 375 (1995) 322-325 or the Affigel blue used by Lopez-Martinez et al., in Curr.Biol. 5 (1995) 791-796 or the heparin agarose particles that they used are less suitable for a pharmaceutical application since they are immunogenic and can cause inflammatory reactions.

The inventors found that the biocompatible and biodegradable carrier collagen described by Kinto et al. for hh-expressing cells is also unsuitable for an optimal local pharmaceutical application of hedgehog proteins. It was found that when the collagen carriers are loaded with hedgehog proteins under physiological conditions (pH ca. 7 and in weak acids (up to pH 4.5)) the majority of the applied hedgehog protein is released from the matrix within minutes. When the loading is carried out under acidic conditions (below pH 4.5) a large amount of the applied hedgehog protein is denatured and bound irreversibly to the carrier.

A pharmaceutical composition of a hedgehog protein is described in EP-A 98101893.0 which is characterized in that the hedgehog protein is bound to a hydrophilic carrier which is biocompatible wherein the carrier is a polymer which binds the hedgehog protein as a negatively-charged carrier due to ionic interactions, the hedgehog protein is not denatured during binding to the carrier, it contains at least 0.1 to 2 negatively-charged residues per monomer under neutral conditions, the charge is in the form of acidic groups, it has an average molecular weight of at least 50,000 Da and contains no agarose.

The object of the invention is to provide a pharmaceutical composition of a hydrophobically modified hedgehog protein containing a biocompatible carrier, wherein the carrier binds the hedgehog protein as an active folded structure and can release it locally in vivo in an active form in a delayed manner. Such formulations are particularly suitable for the repair of bone and cartilage defects and can also be used to repair neuronal defects or for a systemic delivery.

The object is achieved by a pharmaceutical composition of a hydrophobically modified hedgehog protein which is characterized in that this composition contains hydrophobically modified hedgehog proteins and a biodegradable protein or a proteolytic degradation product thereof as a carrier.

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It has surprisingly turned out that hydrophobically modified hedgehog proteins can be released in vivo from such a carrier in a reversible, active and delayed manner without causing immunogenic and/or inflammatory reactions in vivo. The carrier can be a solid carrier such as a sponge or an injectable carrier such as a gel. The preferred biodegradable carrier is collagen or the hydrolysis product gelatin. Other fibrous carrier proteins such as fibrin or elastin are also suitable and can be used as intact protein fibres, as solubilized protein or as partially hydrolysed protein.

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Collagen is understood according to the invention as preferably soluble collagen, insoluble collagen (preferably cross-linked), atelo-collagen or gelatin. According to the invention the collagen can be present as a solid matrix (cross-linked or non-cross-linked lyophilisate or precipitate, as fibres, as a dispersion or as a gel). Recombinantly produced collagen as well as type I or type II collagen and mixtures thereof are particularly suitable. Collagen fibres can for example be prepared according to the British Patent No. 1204438. Soluble collagen can for example be prepared by processes as described in the British Patents No. 990276 and 1184502. The product which is prepared by these methods can be referred to as a microgel and contains a mixture of collagen in various forms of aggregation. Hydrolysed collagen can preferably be obtained by treating collagen with trypsin. This results in a polydisperse mixture of polypeptides with a molecular weight in the range between about 5000 and 70000. The collagen is preferably firstly denatured for example by heat treatment before treatment with trypsin.

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The biodegradable carrier, preferably collagen, is preferably used as a sponge such as for example the collagen sponge Helistat™ from the Integra Life Sciences Company, USA.

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A collagen is preferably used as the carrier matrix, particularly preferably as a soluble or insoluble carrier matrix. The carrier matrix is particularly preferably composed of a combination of the biodegradable carrier and an anionic polysaccharide, such as preferably hyaluronic acid (as well as chemically cross-

linked forms thereof), chondroitin sulfate, polyvinyl sulfate, keratan sulfate, dextran sulfate, pectin, carrageenan and other hydrocolloids, sulfated alginate, dermatan sulfate, alginate, preferably calcium alginate or complexes of protein and polysaccharides such as those described for example in the US patent No. 4,614,794 (Fibracol™, Johnson and Johnson, USA) in which the percentage by weight of the charged polysaccharides is 10-50 %. An insoluble matrix in the sense of the invention means that the matrix does not substantially decompose or significantly dissolve in a neutral buffer solution in vitro within 10-20 hours at room temperature. In this connection it is preferred that the carrier used according to the invention contains less than 50 %, preferably less than 20 % and particularly preferably essentially no amount of a neutral polysaccharide. A combination of collagen with hyaluronic acid with a molecular weight of at least  $10^6$  Dalton, in particular with a molecular weight of  $4 \times 10^6$  Dalton is particularly suitable.

A delayed release is understood according to the invention as a release of the hedgehog protein in a pharmacologically effective dose over a defined period of at least 14 hours. A pharmacologically effect is understood as a neurological effect on nerve cells, a chondrogenesis and/or chondroinduction and preferably osteogenesis and/or osteoinduction as described in Kinto et al., FEBS Letters, 404 (1997) 319-323 for bone induction, by Miao et al. in J.Neurosci. 17 (1997) 5891-5899 for the effect on nerve cells and by Stott et al. in J.Cell Sci. 110 (1997) 2691-2701 for cartilage cell induction.

An enzymatically degradable carrier is preferably used as the carrier which is degraded by enzymes (e.g. proteases) secreted from the cells on which the local in vivo application has occurred. However, the half life of the carrier should be at least 12 h but can be several weeks. If the carrier is composed of a polysaccharide, this carrier is preferably degraded by glycosidases and by hydrolases that are present in and secreted by the cell. However, such a biodegradability of the carrier is not necessary in every case. If the release is carried out to treat osteoporosis or neuronal diseases, a biodegradability is unnecessary. However, such carriers are preferably poorly soluble under physiological conditions and are therefore absorbed by the body over a long period (several weeks to months).

Solutions of hedgehog proteins at high concentrations are required to produce carrier matrices that are coated with hedgehog proteins in such a manner that they exhibit an adequate pharmaceutical efficacy when applied locally. It has turned out

that pharmaceutically suitable carriers coated with hedgehog protein should preferably contain a concentration of the hedgehog protein of 1 - 20 mg/ml carrier and more. Carriers are particularly advantageous which contain hedgehog proteins at a concentration of 10 mg/ml carrier (solution volume, gel volume or sponge  
5 volume) or more. Hedgehog proteins are inherently poorly soluble. A further subject matter of the invention is a process for the production of a carrier matrix coated with hedgehog protein which is characterized in that the carrier matrix is incubated with a hedgehog protein solution at a concentration of 3 mg/ml which contains arginine or argininium ions and the carrier matrix coated in this manner  
10 is isolated.

Such solutions are suitable for producing carrier matrices which contain hedgehog proteins in pharmaceutically effective concentrations and are suitable for pharmaceutical applications. Hence a further subject matter of the invention is a  
15 collagen carrier which contains 3 mg hydrophobically modified hedgehog protein or more, preferably 10 mg or more per ml carrier matrix and arginine or argininium ions (preferably argininium sulfate). The concentration of arginine is

test). In this method a mouse fibroblast cell line is cultured in a medium which contains foetal calf serum. Subsequently sterile filtered sample is added, the cells are  
25 lysed after ca. 5 days and alkaline phosphatase is determined in the cell lysate by means of the cleavage of a chromogenic substrate (pNP, p-nitrophenol) (J. Asahina, Exp. Cell. Res. 222 (1996) 38 - 47 and T. Nakamura (1997)).

A hydrophobically modified (lipophilized) hedgehog protein is understood by the  
30 invention as a lipophilized secreted signal protein which is responsible for the formation of numerous structures in embryogenesis. Sonic, indian or desert hh are particularly preferably used (Fietz M. et al., Development (Suppl.) (1994) 43-51). The processed form (N-terminal mature domain) of sonic hh protein described in EMBL data bank under the No. L38518 is preferably used. Proteins of the hedgehog  
35 family exhibit a pronounced homology in their amino acid sequence which is why it is also preferable to express those nucleic acids which code for hedgehog proteins

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which are 80 % or more homologous with the above-mentioned sequence of sonic hedgehog protein.

Such a lipophilization is preferably achieved by chemical modification. Such a hedgehog conjugate preferably contains an additional polypeptide that is covalently bound (preferably at the C-terminus and/or N-terminus) and is composed of 10 - 30 preferably hydrophobic amino acids and/or those amino acids which form transmembrane helices. The additional polypeptide particularly preferably contains 2 - 12 lysines and/or arginines but no polyhistidine part that would be suitable for purifying the conjugate on a Ni chelate column. It is also preferable to covalently bind (preferably at the C-terminus and/or N-terminus) 1 - 4 aliphatic, saturated or unsaturated hydrocarbon residues with a chain length of 10 - 24 C atoms or steroids with a lipophilic (hydrophobic) action. Furthermore it is preferred to covalently couple hydrophobic thio compounds, such as in particular thiocholesterol and thioalkanes, thioalkenes, to hh proteins via a disulfide bridge formed oxidatively (preferably at the C-terminus and/or N-terminus and in this case on the N-terminal cysteine).

The protein is hydrophobized by such lipophilizing residues which improves its interaction with lipid membranes of eukaryotic cells, in particular of mammalian cells.

Consequently a lipophilized protein according to the invention is understood as a hydrophobized protein which has an increased surface hydrophobicity compared to an unmodified protein which increases its affinity for apolar molecules or amphiphiles. The increase in the degree of lipophilicity of the protein can be measured by the degree of integration in a lipid layer as described for example by Haque, Z. et al., J.Agric.Food Chem. 30 (1982), 481. Methods for the hydrophobic (lipophilizing) modification of proteins are for example described by Haque, Z. et al., J.Agric.Food Chem. 31 (1983) 1225-1230; Webb, R.J. et al., Biochemistry 37 (1998) 673-679; Hancock, J.F., Cell 63 (1990) 133-139; A Practical guide to membrane protein purification, Ed. G.v. Jagow, Hermann Schägger (1994), (chapter 16, pages 535-554).

Such hydrophobically modified hedgehog proteins are described for example in the European Patent Applications No. 98116733.1 and 98107911.4.

5 The human sonic hedgehog precursor protein is composed of the amino acids 1 - 462 of the sequence described in the EMBL databank under No. L38518. The amino acids 1 - 23 represent the signal peptide, the amino acids 24 - 197 represent the mature signal domain, the amino acids 32 - 197 represent the signal domain shortened by eight amino acids and the amino acids 198 - 462 represent the autoprocessing C-terminal domain after autoproteolytic cleavage.

10 The pharmaceutical composition according to the invention contains a pharmacologically effective dose of the hh protein and can be administered locally. It is preferable to use the proteins according to the invention in combination with other proteins of the hedgehog family or bone growth factors such as bone morphogenetic proteins (BMPs) (Wozney et al., Cell.Mol.Biol. of Bone, Bone Morphogenetic Proteins and their Gene Expression (1993) Academic Press Inc., 131-167) or parathyroid hormones (Karablis et al., Genes and Development 8 15 (1994) 277-289) or insulin-like growth factors (IGF-I or II) or transforming growth factors (TGF- $\beta$ ).

20 The pharmaceutical composition is produced by incubating the lipophilized hedgehog protein with the carrier or with the carrier containing collagen at a pH value of 4.5 or more. The incubation is preferably carried out at a neutral pH value (pH 6-8) and preferably in a buffered solution. At pH 4.5 or higher pH values, the lipophilized hedgehog protein binds to the carrier by hydrophobic interactions. If the carrier matrix additionally contains a hydrophilic carrier which can bind proteins by means of ionic interactions (for example an anionic polysaccharide), the hedgehog protein can then also interact with this part of the carrier by means of ionic interactions. For this reason the ratio of collagen to the hydrophilic carrier moiety can vary over a wide range. However, it is preferable that the ratio of collagen moiety to hydrophilic carrier moiety is 1.5:1 or more.

30 Furthermore it is preferable for the production of the pharmaceutical composition to add auxiliary substances such as a sugar (mannitol, lactose, glucose, sucrose, trehalose, preferably 20-100 mg/ml) or amino acids such as glycine or arginine, oxidation inhibitors such as methionine as well as antioxidants such as EDTA, citrate, polyethylene glycol (1 - 10 % by weight), detergents, preferably non-ionic detergents 35 (preferably 0.005 - 1 % by weight) such as polysorbates (Tween<sup>®</sup>20 or Tween<sup>®</sup>80) or polyoxyethylenes, anti-inflammatory agents, local anaesthetics, antibiotics and/or stabilizers such as lipids, fatty acids and glycerol.

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In a further preferred embodiment a pharmaceutical composition of the hedgehog protein according to the invention containing suramin is preferred and this can be used advantageously.

The pharmaceutical composition can contain additional pharmaceutical auxiliary substances.

In a preferred embodiment the pharmaceutical composition contains the lipophilized hedgehog protein at a concentration of 0.1 - 100 mg/ml.

In a preferred embodiment the pharmaceutical composition additionally contains a pharmaceutically acceptable buffer which is biocompatible, preferably in the range between pH 4 and pH 10, particularly preferably in the range between pH 6 and 8. The pH value of the pharmaceutical composition should be advantageously greater than pH 4 in order to prevent denaturation and detachment of the zinc complexed in the hedgehog protein. The concentration of the buffer is preferably 1-500 mmol/l, in particular 5-150 mmol/l and particularly preferably 10-100 mmol/l. In a suitable embodiment 300 mmol/l potassium phosphate buffer, pH 6.0 or 300 mmol/l arginine chloride, 10 mM potassium phosphate, pH 6.0 is used as a buffer.

The following examples, publications and the figures further elucidate the invention, the protective scope of which results from the patent claims. The described methods are to be understood as examples which still describe the subject matter of the invention even after modifications.

#### Description of the Figures:

**Fig. 1:** In vitro release of hydrophobically modified shh from a collagen matrix (Helistat™).

**Fig. 2:** In vitro release of dipalmityl-shh from a collagen/alginate matrix (Fibracol™).

## Examples

### Example 1

In vitro release of hydrophobically modified hedgehog proteins from a collagen matrix (Helistat™)

0.1 ml aliquots of solutions of variously modified hedgehog proteins (palmityl-shh = 1xC16-shh, dipalmityl-shh = 2xC16-shh or cholesteryl-shh = Chol-shh) are applied dropwise to collagen sponges (Helistat™, Integra Life Sciences, USA) with dimensions of 10 x 10 x 3 mm. The loaded carriers are frozen (-70°C), lyophilized and analysed. For this the sponges are incubated at 37°C in a suitable volume of PBS. The amount of released hh is determined by means of rp-HPLC (see Fig. 1).

The analysis of the in vitro release kinetics shows that Shh is released in a delayed manner or in small amounts from the collagen matrix over a period of 20 h (Fig. 1).

It can be seen that in vitro only ca. 20 % of the loaded 2xC16-shh is released under the stated conditions. The remaining 80 % of the applied protein can be released in vivo by biodegradation of the carrier. This situation can be simulated in vitro by an enzymatic degradation of the collagen carrier with recombinant collagenase. For this a collagen sponge loaded with 2xC16-shh which had been incubated for 14 h under release conditions (see above) was transferred into 490 µl buffer (50 mM HEPES-NaOH, 800 mM NaCl, 0.1 % Tween, pH 7.4) and 10 µl recombinant collagenase I (1 mg/ml, in 10 mM Tris-Cl, 1 mM CaCl<sub>2</sub>, pH 7.4) was added. After incubation for 2 h at 37°C the released hedgehog protein is analysed by means of reversed phase HPLC and in an in vitro activity test (induction of alkaline phosphatase in C3H10T1/2 cells). It is clear from this analysis that the non-released fraction of 2xC16-shh (see above) that remains in the collagen carrier under release conditions can be released by means of collagenase digestion and its activity is not lost in this process (ca. 80 % recovery).

**Example 2:**

**In vitro release of hydrophobically modified hedgehog protein (dipalmityl-shh) from a collagen-alginate matrix (Fibracol™)**

5 0.05 ml aliquots of a solution of dipalmityl-shh (1 mg/ml) are added dropwise to collagen-alginate sponges (Fibracol™; Johnson & Johnson, USA) of approximately 10 x 10 x 3 mm size. The loaded carriers are frozen (-70°C), lyophilized and analysed. For this the sponges are incubated at 37°C in a suitable volume of PBS. The amount of released 2xC16-shh is determined by means of rp-HPLC (see Fig.  
10 2).

15 It can be seen that in vitro only ca. 10 % of the loaded 2xC16 is released under the stated conditions. The remaining 90 % of the applied protein can be released in vivo by biodegradation of the carrier. This situation can be simulated in vitro by a partial enzymatic degradation of the carrier with recombinant collagenase. For this the carriers loaded with 2xC16-shh which had been incubated under release conditions (see above) were transferred into 490 µl buffer (50 mM HEPES-NaOH, 800 mM NaCl, 0.1 % Tween, pH 7.4) and 10 µl recombinant collagenase I (1 mg/ml, in 10 mM Tris-Cl, 1 mM CaCl<sub>2</sub>, pH 7.4) was added. After incubation for 2  
20 h at 37°C the released hedgehog protein is analysed after centrifugation by means of reversed phase HPLC. It is clear from this analysis that the non-released fraction of 2xC16-shh that remains in the collagen-alginate carrier under release conditions can be released by means of collagenase digestion (ca. 80 % recovery; Fig. 2).

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